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SHORT COMMUNICATION

GLYCOSIDASES FROM MALTED BARLEY*

EARL D. MITCHELL and JEFF NEWMANN

Department of Biochemistry, Agricultural Experiment Station, Oklahoma State University, Stillwater, Oklahoma 74074, U.S.A.

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Abstract—An α-mannosidase, which is able to hydrolyse *p*-nitrophenyl-α-mannoside and yeast mannan, has been partially purified from malted barley. The barley extract contained activities of α-galactosidase, β-galactosidase, α-glucosidase, β-glucosidase and *N*-acetylglucosaminidase as measured by hydrolysis of their various *p*-nitrophenyl glucosides. The α-mannosidase is shown to liberate mannose from yeast mannan and ovalbumin. The fact that no other sugar was released indicates that this enzyme is an exo-enzyme and that terminal α-mannosidic linkages exist in these carbohydrate moieties.

INTRODUCTION

THE α-MANNOSIDASE (α-D-mannoside mannohydrolase, E.C. 3.3.2.14), and α-galactosidase (α-D-galactoside galactohydrolase, E.C. 3.2.1.22) of barley are potential glycoprotein hydrolases. The monosaccharides most commonly found in glycoproteins are D-mannose, D-galactose, *N*-acetylglucosamine and L-fucose.

In this paper, we report on a highly purified preparation of α-mannosidase obtained from malted barley. Classical sources for this hydrolase are almond emulsin and jack bean meal. Malted barley along with wheat is a cheaper source and commercially available in large quantities. α-Mannosidase, devoid of other glycosidase activities is a useful tool for structural studies on glycoprotein, glycolipids, and oligosaccharides.

RESULTS AND DISCUSSION

The crude extract (Table 1) was assayed for glycosidase activities. Following ammonium sulfate saturation (Fig. 1), a Sephadex G-200 column (Fig. 2) followed by a DEAE-cellulose column (Fig. 3) were used for purification. The highest purification was 1130; however, usually the purification factor was 800–900 fold. After gel filtration and ion exchange chromatography, the enzyme preparation was usually stored as it came off the DEAE-cellulose column at 0–5°. No appreciable loss of activity was detected during a month in storage. Before use, the enzyme solution was concentrated by vacuum dialysis.

TABLE 1. PURIFICATION OF α-MANNOSIDASE FROM BARLEY

Fraction	Steps	Specific activity (units/mg protein × 10^3)	Yield (%)	Purification factor
1	Crude extract	2·2	100	1
2	$(NH_4)_2SO_4$ precipitate 30–55% saturation	50	60	23
3	G-200 Sephadex	444	30	202
4	DEAE-cellulose	2500	11	1130

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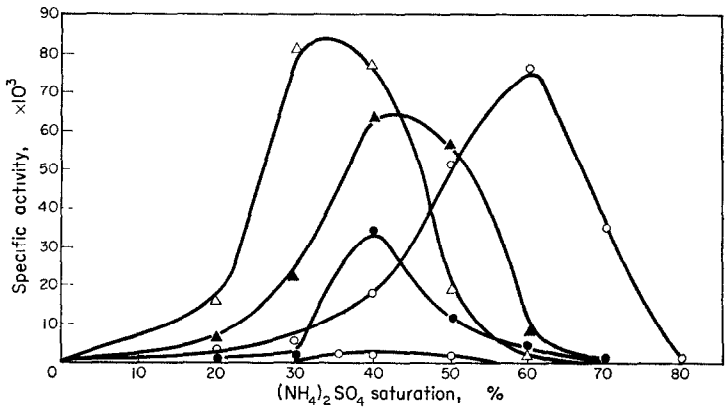

FIG. 1. PER CENT $(NH_4)_2SO_4$ FRACTIONATION OF SEVERAL GLYCOSIDES VS. SPECIFIC ACTIVITIES. The crude extract was treated with $(NH_4)_2SO_4$ to obtain 20% saturation (114 g/l.). The precipitate was resuspended and assayed for glycosidase activities. The supernatant was treated with additional $(NH_4)_2SO_4$ to obtain precipitates at 30, 40, 50, 60, 70, and 80% saturation followed by glycosidase activity and protein concentration measurements of each resuspended precipitate. ●—α-mannosidase activity; ○—β-glucosidase; ▲—β-galactosidase; △—α-galactosidase; ○—β-N-acetylglucosaminidase.

The α-mannosidase was free from α- and β-galactosidase, β-*N*-acetyl glucosaminidase and α- and β-glucosidase as determined by using *p*-nitrophenyl glucosides as substrates. When the enzyme was examined by polyacrylamide gel, several protein bands were observed, an indication that the enzyme was not homogeneous. The α-mannosidase was stable to freezing and thawing. When the enzyme was prepared in 0·01 M acetate buffer pH 5·5, and stored for 3 months at 4°, there was virtually no loss of activity. Heating the malted barley extract to 70° for 15 min destroyed the activities of the glycosidases. α-Mannosidase shows maximum activity at 50° and a very sharp loss of activity at 60°.

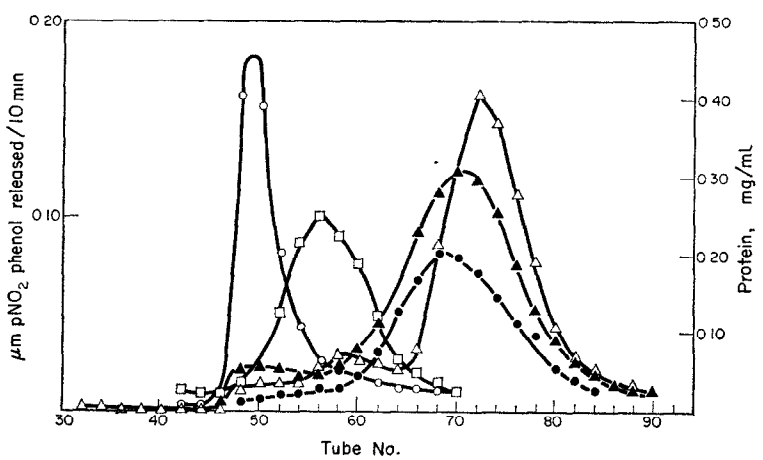

FIG. 2. SEPHADEX G-200 FILTRATION OF α-MANNOSIDASE PREPARATION. ○—α-Mannosidase activity; □—β-*N*-acetylglucosaminidase; ●—β-galactosidase; ▲—β-glucosidase; △—protein.

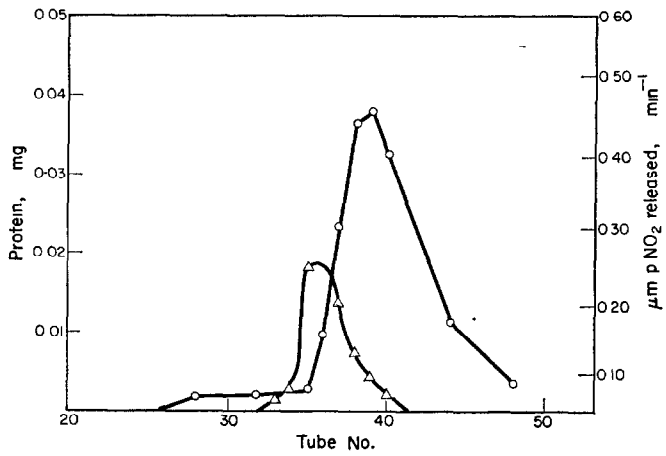

FIG. 3. DEAE-CELLULOSE CHROMATOGRAPHY OF α-MANNOSIDASE PREPARATION.
△—Absorbtion at 420 nm for α-mannosidase activity; ○—mg protein 3 ml fractions were collected.

Effect of Substrate Concentration

The effect of varying substrate concentration on the reaction rate at pH 4·6 (0·05 M citrate buffer) with *p*-nitrophenyl α-mannoside was studied. The Lineweaver–Burk[1] plot was linear and shows the rate of hydrolysis of *p*-nitrophenyl-α-mannoside was $1{\cdot}1 \times 10^{-3}$ M.

Effect of pH

The optimum for barley α-mannosidase in Tris-acetate buffer occurs at pH 3–5, which is similar to the value reported by Li[2,3] for the jack bean meal enzyme.

Mannose Liberation from Various Glycoproteins

α-Mannosidase from barley liberated mannose from ovalbumin and yeat mannan. Like the enzyme from jack bean meal, only when a terminal α-mannosidic linkage was available was mannose released from the glycoprotein. The rate of mannose release varied between ovalbumin and mannan, since the rate would be dependent upon the polypeptide moiety and the sequential length of the oligosaccharide chain in the glycoprotein.

In addition to properties of hydrolysis and glycosyl transfer, glycosidases are known to catalyse the condensation of monosaccharides to form oligosaccharides.[3] The synthetic properties of α-mannosidase was investigated by incubation at 37° for 4 hr of 1·0 ml of enzyme solution containing 200 units of the enzyme with 1·0 ml of mannose (1 mM), both solutions being in 0·01 M acetate buffer pH 5·5. After incubation the enzyme was inactivated by heating in a steam bath for 10 min, the precipitated protein was removed by centrifugation, and the supernatant was concentrated *in vacuo* and passed through a Dowex 50 ion exchange column. Five μl of the resulting effluent was examined for sugars by paper chromatography (see Experimental).

In addition to α-mannosidase and α-galactosidase, malted barley also contains several other glycosidases, i.e. α- and δ-glucosidases, β-galactosidase and *N*-acetylglucosaminidase.

[1] H. LINEWEAVER and D. BURK, *J. Am. Chem. Soc.* **56**, 658 (1934).
[2] YU-TEH LI, *J. Biol. Chem.* **241**, 1010 (1966).
[3] YU-TEH LI, *J. Biol. Chem.* **242** 5474 (1967).

The activities of α-glucosidase were very low ($2{\cdot}6 \times 10^{-3}$ units/mg) when assayed with *p*-nitrophenyl glucosides as substrates whereas the activities of the other glycosides were greater ($21\text{–}58 \times 10^{-3}$ units/mg).

Unlike the jack bean meal enzyme, the α-mannosidase from malted barley did not lose activity after storage at pH 4·6. The *p*-nitrophenyl-α-mannoside substrate saturation curve and the Lineweaver-Burk plots indicate an affinity of the enzyme for this synthetic substrate. When ovalbumin was incubated with the purified α-mannosidase, a large amount of free mannose was liberated which is in accord with the structure of the carbohydrate moiety as determined by Montgomery.[4] When the enzyme was incubated with yeast mannan only mannose was detected. These results are similar to the results of Li[3] with studied on the α-mannosidase from jack bean meal.

EXPERIMENTAL

Materials. Malted barley was a gift from the Minnesota Malting Company, Cannon Falls, Minnesota, Kurth Malting Corporation, Milwaukee and Bio-Technical resources, Monitowoc, Wisconsin.

Assay procedure. Various *p*-nitrophenyl glucosides were used as substrates. When *p*-nitrophenyl-α-D-mannoside and *p*-nitrophenyl-α-D-galactopyranoside were substrates, the incubation mixture contained, in a total volume of 2 ml, sodium citrate buffer pH 4·6, 67 μmol and 0·5 ml of the properly diluted enzyme solution. The mixture was incubated for 10 min at 30° and the reaction was terminated by adding 3 ml of 5% Na_2CO_3. The liberated *p*-nitrophenol was quantitatively determined spectrophotometrically at 420 nm ($\epsilon = 2{\cdot}74 \times 10^4\ M^{-1}\ cm^{-1}$). A unit of enzyme is defined as the amount that releases 1 μmol of *p*-nitrophenyl per min and specific activity is expressed as the number of units per mg of protein. The sum of absorbance developed when enzyme and substrate were incubates separately was subtracted from the complete reaction value. In most cases the substrate absorbance values were 0·001–0·005.

Ammonium sulfate fractionation. The crude extract (Table 1) was treated with $(NH_4)_2SO_4$, the fractions collected by centrifugation and suspended in NaOAc buffer, 0·01 M/pH 5·5. The solutions were dialysed overnight against the same buffer and assayed for glucosidase activities (Fig. 1). For α-mannosidase preparation a 30–55% $(NH_4)_2SO_4$ saturation fraction (176–351 g/l.) was obtained.

G-200 Sephadex fractionation. The solution (5 ml, 0·12 g protein) was applied to a G-200 Sephadex column (60 × 2·5 cm) equilibrated with 0·01 M acetate buffer pH 5·5 and eluted with the same buffer in 3 ml fractions at a flow rate of 17 ml/hr (Fig. 2). The most active fractions of mannosidase were pooled, dialysed and lyophilized. This procedure was repeated several times in order to process the entire 30–55% $(NH_4)_2SO_4$ precipitate (20 ml, 0 5 g protein).

DEAE-cellulose. The combined concentrated fraction dissolved in 0·01 M acetate buffer was applied to a DEAE-cellulose column (2·5 × 60 cm) that had been equilibrated with 0·01 M acetate buffer pH 5·5. The column was eluted with a linear gradient from 0·01 M acetate fo 0·1 M acetate and 0·6 M NaCl at pH 5·5 (Fig. 3). For the α-mannosidase the pH optimum was determined by dissolving the substrate in 0·1 M Tris-acetate. The desired pH was obtained by the addition of N HCl or N NaOH. The reaction was terminated and absorbance was measured in the usual manner.

Mannose release from glycoproteins and mannan. Either ovomucoid, ovalbumin or mannan, 1 mg each, was incubated with 5 units of α-mannosidase in 2 ml of 0·01 M, citrate buffer pH 4·6 for 8 hr at 37°. The incubation was terminated by heating in a steam bath for 5 min. After the precipitated protein was removed by centrifugation, the solution was concentrated *in vacuo* (max. 45°) to 0 2 ml and 60 μl aliquots were applied to Whatman No. 1 paper. Qualitative identification of mannose release in the enzymatic digest was by descending chromatography with pyridine–EtOAc–HOAc–H_2O (5:5:1:3). Reducing sugars were detected by spraying the paper with α-aminobiphenyl or by dipping the paper in $AgNO_3$ as described by Benson *et al.*[5] All of the operations were conducted between 0 and 4° unless otherwise stated. Protein was determined by the method of Lowry *et al.*[6]

Preparation of crude extract. Malted barley was ground with a Wiley mill through a 30 mesh screen. A 144 g quantity of ground barley was extracted with 600 ml of 0·01 citrate buffer pH 5·5 for 3 hr at 5°. The extract was then centrifuged at 14 000 *g* 20 min, the supernatant being termed 'crude extract'. This crude extract was assayed for various glycosidase activities.

[4] R. MONTGOMERY, Y. C. LEE and Y. C. WU, *Biochem.* **4** 566 (1965).

[5] A. A. BENSON, J. A. BASSHAM, M. CALVIN, A. G. HALL, H. C. HIRSCH, LYNCH V. KAWAGUCHI and N. E. TOLBERT, *J. Biol. Chem.* **196,** 703 (1952).

[6] O. H. LOWRY, N. J. ROSEBROUGH, A. L. FARR and R. I. RANDALL, *J. Biol. Chem.* **193,** 265 (1951).

Key Word Index—Hordeum vulgare; Gramineae; malted barley; glycosidases; α-mannosidase.